

## Expression of Gonococcal Transferrin-Binding Protein 1 Causes *Escherichia coli* To Bind Human Transferrin

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**The gene for gonococcal transferrin-binding protein 1 (TBP1) was cloned behind an inducible promoter in *Escherichia coli*. The resultant strain was capable of binding human transferrin with the same specificity as that of the gonococcus. *E. coli* expressing TBP1 did not internalize transferrin-bound iron or grow on transferrin as a sole iron source.**

The pathogenic *Neisseria* species are capable of utilizing human transferrin (Tf) as their sole source of iron in the absence of siderophore production (9). Tf receptor function in these organisms has been characterized as saturable and specific for human Tf (2, 7, 14, 16). Two Tf-binding proteins, TBP1 and TBP2, are potential components of a Tf receptor. Both TBP1 and TBP2 are expressed preferentially under iron-restricted growth conditions (3, 12, 13), and each binds human Tf specifically (3, 12, 13). The gene encoding gonococcal TBP1, *tbpA*, was recently cloned and sequenced (3). Transposon mutagenesis of *tbpA* generated gonococcal mutants that were incapable of utilizing Tf as a source of required iron and bound less Tf to their cell surfaces. The predicted protein sequence of TBP1 was homologous to that of the TonB-dependent outer membrane receptors of *Escherichia coli*. These observations were consistent with the hypothesis that TBP1 is a Tf receptor in the gonococcus. However, they did not eliminate the possibility that TBP2 was also required for Tf receptor activity. In addition, it remained possible that mutations in *tbpA* distorted the membrane framework, disturbing Tf receptor function without altering the receptor itself (3).

In an effort to assess the role of TBP1 in transferrin utilization, we cloned the entire coding region of the gonococcal *tbpA* gene behind an inducible promoter in *E. coli*, which normally does not bind Tf. The resultant strain was analyzed for TBP1 expression, Tf binding, Tf-Fe uptake, and growth on Tf as a sole iron source.

**Construction of a complete *tbpA* gene.** The *tbpA* gene was previously cloned in two pieces, contained in clones pUNCH405 and pUNCH115 (Fig. 1). An intact *tbpA* gene was constructed in pUP1 (4) by ligation of the insert in pUNCH405 with the insert in pUNCH115 at the downstream *Cla*I site. A subclone, pUNCH411, was constructed by ligating the *Hind*III-to-*Xba*I fragment into pBluescript SK(+) (Stratagene). This clone did not express TBP1 when in the *E. coli* strain DH5 $\alpha$ MCR (Bethesda Research Laboratories) (data not shown). pUNCH412 (Fig. 1) was constructed by ligating the *Hind*III-to-*Xba*I fragment from pUNCH411 into the expression vector pET-11 (15) (available from Novagen). In this construct, a T7lac promoter was located upstream of the *Hind*III site and a T7 terminator was located downstream of the *Xba*I site. pUNCH412 was transformed into the *E. coli* strain BL21(DE3)pLysE (15), which

conditionally expresses T7 polymerase in the presence of IPTG (isopropylthiogalactoside).

**Time course of expression and localization of TBP1 in *E. coli*.** BL21(DE3)pLysE containing pUNCH412 (FS1033) or the vector pET-11 (FS1034) was grown in LB medium (8) and induced with 1 mM IPTG for various amounts of time. As a control, gonococcal strain FA19 (9) was grown in GCB medium (Difco) containing Kellogg's supplements I and II (5) and iron starved by addition of the chelator deferoxamine mesylate (Desferal; CIBA Pharmaceuticals) at a concentration of 50  $\mu$ M for 3 h. At each time point, an aliquot was removed and approximately  $6.5 \times 10^7$  cells were pelleted and solubilized. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6), transferred to nitrocellulose, and probed with polyclonal antiserum raised against purified TBP1 (3). Also, approximately  $10^7$  cells from each time point were spotted onto nitrocellulose and assessed for their ability to bind human Tf (3). FS1034 did not express TBP1, whereas FS1033 expressed increasing amounts of TBP1 as exposure time to the inducer increased (Fig. 2A). The molecular weight of TBP1 expressed in *E. coli* was identical to that of the protein expressed by FA19 under iron-deficient conditions (Fig. 2A). Immunoreactive proteolytic breakdown products were also apparent after addition of the inducer.

Expression of TBP1 by FS1033 was sufficient to cause whole cells to bind human Tf, while FS1034 bound no Tf (Fig. 2B). As expected, expression of this binding phenotype was also regulated by IPTG exposure. Binding of Tf to whole cells and localization of TBP1 in the sarcosyl-insoluble membrane fraction (data not shown) indicated that TBP1 was apparently properly processed and functional in *E. coli*.

Although very little TBP1 was detectable in whole-cell lysates of FS1033 without induction (Fig. 2A, lane 3), uninduced cells exhibited significant Tf binding (Fig. 2B, column 1). The increase in TBP1 expression during the first 30 min of induction was much greater than the corresponding increase in whole-cell Tf-binding phenotype. These discrepancies may be due to the inability of all of the expressed TBP1 to be processed and localized on the cell surface or saturation of the horseradish peroxidase detection system.

**Specificity of Tf-binding activity.** To determine whether Tf binding by TBP1-expressing *E. coli* was specific for human Tf, a competitive dot blot was performed using cells that had been induced with IPTG for 30 min. In a control blot, FA19 was spotted to nitrocellulose after 3 h of iron starvation. Fourfold dilutions of unlabelled mammalian transferrins

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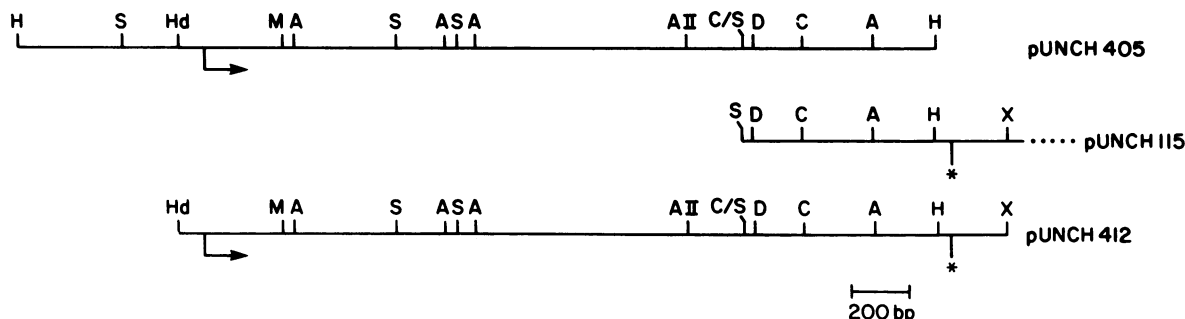


FIG. 1. Restriction maps of *tbpA*-specific clones. Arrows indicate the position and direction of the open reading frame corresponding to TBP1. Asterisks indicate the end of the *tbpA* gene. The following designations indicate sites of restriction endonuclease recognition: A, *AvaI*; AII, *AvaII*; C, *ClaI*; D, *DraI*; H, *HincII*; Hd, *HindIII*; M, *MluI*; S, *Sau3AI*; and X, *XbaI*. The *ClaI* site which is overlapped by a *Sau3AI* site (indicated by C/S) is not cut in *E. coli*. The 2.8-kb region downstream of the *XbaI* site in pUNCH115 is shown as a dotted line.

were mixed with a constant concentration of labelled human Tf (0.4  $\mu$ g/ml, 2.8 nM) and allowed to bind to whole-cell dot blots for 1 h. Blots were washed and developed (3). *E. coli* expressing TBP1 (FS1033) bound human Tf specifically, since binding was not displaced by unlabelled mammalian Tfs (Fig. 3). These findings indicated that TBP1 is a specific Tf receptor requiring no other gonococcal factors to impose the specificity.

To address whether TBP1 expression allowed Fe uptake from and growth on Tf, an enterochelin-deficient *E. coli* strain which conditionally expressed T7 polymerase was constructed. This was necessary because enterochelin is capable of deferring Tf (1), and an enterochelin-producing strain was able to grow in the presence of Tf (data not shown). Isogenic *tonB*<sup>+</sup> and *tonB* mutant strains (RWB18-60 and KDF570, respectively) that were incapable of synthesizing and utilizing enterochelin were obtained from Phillip Klebba (11). These were lysogenized with  $\lambda$ DE3 (phage lysate and protocol obtained from Novagen), which encodes T7 polymerase under the control of an IPTG-responsive promoter (15), resulting in strains FS1027 (*tonB*<sup>+</sup>) and FS1028 (*tonB* mutant). These strains were then transformed

with pUNCH412 and the vector pET-11, resulting in strains FS1029 (*tonB*<sup>+</sup>, pUNCH412), FS1030 (*tonB*<sup>+</sup>, pET-11), FS1031 (*tonB* mutant, pUNCH412), and FS1032 (*tonB* mutant, pET-11). None of the above strains, regardless of the TonB phenotype, took up Fe from or grew on Tf as a sole Fe source, although TBP1 was expressed in IPTG-induced strains as expected (data not shown). These results indicated that expression of TBP1 allowed binding of human Tf but was not sufficient for Fe uptake from or growth on Tf. This suggests that other neisserial proteins which *E. coli* did not provide are required for Tf utilization. These may include TBP2, the function of which is unclear, and FBP, the periplasmic ferric-binding protein (10).

We conclude that gonococcal TBP1 is a Tf receptor and that it does not require the presence of TBP2 to specifically bind human Tf to the cell surface. However, additional gene products, perhaps including TBP2, are required to release or transport Fe from Tf. It is possible that Tf binding affinities would be altered if both TBP1 and TBP2 were simultaneously expressed in *E. coli*; we did not quantitate Tf affinities in these experiments. It may be possible to reconstruct a fully functional Tf receptor and Fe uptake system in

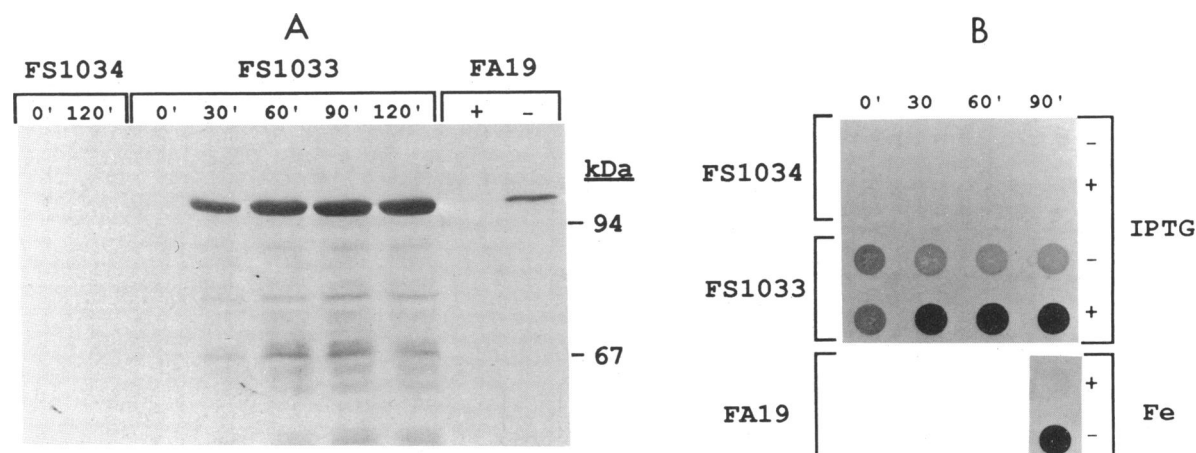


FIG. 2. Time course of expression of TBP1 in *E. coli*. (A) Western blot (immunoblot) probed with anti-TBP1 antiserum. *E. coli* FS1033 and FS1034 were induced with IPTG, and aliquots for whole-cell lysate preparation were removed after the time periods indicated above each lane. The last two lanes contain whole-cell lysates from gonococcal strain FA19 grown under iron-sufficient (+) or iron-deficient (-) conditions. Positions of molecular size standards are indicated at the right in kilodaltons. (B) Dot blot probed with horseradish peroxidase-Tf (1  $\mu$ g/ml). Strains are the same as for panel A. The time of induction is indicated at the top of blot. Strains were grown with (+) or without (-) IPTG or Fe as indicated at the right.

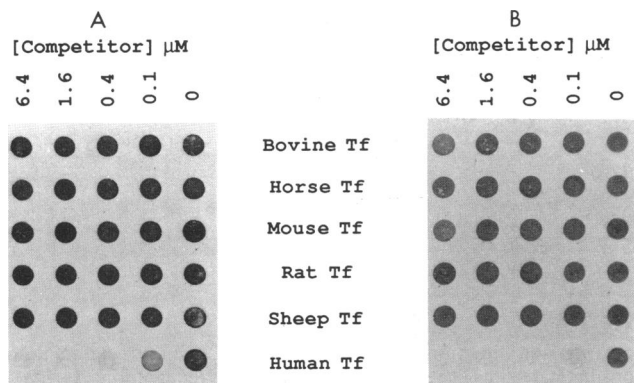


FIG. 3. Specificity of Tf-binding activity. Blots were probed with a mixture of horseradish peroxidase-labelled Tf (0.4  $\mu$ g/ml, 2.8 nM) and unlabelled mammalian Tfs at the concentrations indicated above the blots. (A) Gonococcal strain FA19 grown under iron-deficient conditions; (B) *E. coli* FS1033 grown with IPTG to induce TBP1 expression.

a heterologous host such as *E. coli* by expressing multiple recombinant gonococcal genes.

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